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The Rh Polypeptide Is a Major Fatty Acid-acylated Erythrocyte Membrane Protein*

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The erythrocyte Rh antigens contain an M_r = 32,000 integral protein which is thought to contribute in some way to the organization of surrounding phospholipid. To search for possible fatty acid acylation of the Rh polypeptide, intact human erythrocytes were incubated with [^{3}H]palmitic acid prior to preparation of membranes and sodium dodecyl sulfate-polyacrylamide gel electrophoresis and fluorography. Several membrane proteins were labeled, but none corresponded to the glycophorins or membrane proteins 1–8. An M_r = 32,000 band was prominently labeled on Rh (D)-negative and -positive erythrocytes and could be precipitated from the latter with anti-D. No similar protein was labeled on membranes from Rh_{mod} erythrocytes, a rare phenotype lacking Rh antigens. Labeling of the Rh polypeptide most likely represents palmitic acid acylation through thioester linkages. The ^{3}H label was not extracted with chloroform/methanol, but was quantitatively eluted with hydroxylamine and cochromatographed with palmitohydroxamate and free palmitate by thin layer chromatography. The fatty acid acylations occurred independent of protein synthesis and were completely reversed by chase with unlabeled palmitate. It is concluded that the Rh polypeptide is fatty acid-acylated, being a major substrate of an acylation-deacylation mechanism associated with the erythrocyte membrane.

The human erythrocyte Rh antigens are of great clinical importance, but have only recently become understood on a molecular level (see reviews, Refs. 1 and 2). An M_r = 32,000 integral protein ("Rh polypeptide") is the membrane site of the Rh antigens (3, 4). The Rh polypeptide is unusual in that it contains no detectable carbohydrate (5) and is associated with the membrane skeleton (6–8). The Rh polypeptide has recently been purified (9, 10); the NH_2-terminal amino acid sequence has been determined (11, 12); internal polymorphisms have been identified (12–14). Nevertheless, a physiological role for the Rh antigen and polypeptide have not yet been identified.

Covalent attachment of lipid could explain several puzzling characteristics of the Rh polypeptide. Efforts to solubilize the Rh polypeptide during purification were impeded by an extreme degree of hydrophobicity (9, 10). Several observations indicate that the Rh antigen may be a complex of lipid specifically associated with the Rh polypeptide (15–18), and rare individuals lacking all Rh antigens (Rh_{null} (19)) express secondary defects (20, 21) resulting from abnormal membrane bilayer phospholipid organization (22). Recent work has demonstrated that fatty acid acylation of some membrane proteins is a specific biological event (see review, Ref. 23), and certain membrane proteins of embryonic avian erythrocytes were found to undergo post-translational fatty acid acylation (24). This report demonstrates palmitic acid acylation of the Rh polypeptide and partially characterizes the mechanism by which this post-translational event occurs.

EXPERIMENTAL PROCEDURES

[^{3}H]Palmitic Acid Labeling—The procedure was derived from a published method (24). Freshly drawn human erythrocytes were washed free of other cells and suspended 1:10 in 2 ml of minimum essential medium (Gibco) containing 5 mM pyruvate, 0.1 mM L-amino acids, pH 7.4, to which 2 mCi of [9, 10 ^{3}H]palmitic acid (29 Ci/mmol, Du Pont-New England Nuclear) was added. The cells were shaken overnight at 37 °C, then washed in chilled 0.15 M NaCl, 10 mM $NaPO_4$, 1 mM NaEDTA, pH 7.4. Membranes were prepared (25) and electrophoresed into 14% SDS[1]-PAGE gels (26). The gels were Coomassie-stained, soaked with enhancing solution (Amplify, Amersham), and exposed to autoradiographic film (Kodak, XAR) for up to 1 week. Immunoprecipitations were performed basically as described (10) with Rh (D) immune globulin (Cutter) or nonimmune globulin added to the tubes during the [^{3}H]palmitic acid labeling.

Characterization of Acylations—Membranes prepared from [^{3}H] palmitic acid-labeled erythrocytes were depleted of noncovalently associated lipid by repeated extractions with $CHCl_3/CH_3OH/H_2O$ (2:2:1.8) (27), and the delipidated membranes were incubated in 0.5 M hydroxylamine, pH 9, as described (28) prior to SDS-PAGE. In other experiments, Rh polypeptides immunoprecipitated from [^{3}H] palmitic acid-labeled erythrocyte membranes were repeatedly extracted with $CHCl_3/CH_3OH/H_2O$, and approximately 45% of the immunoprecipitated ^{3}H was thereby extracted (40% removed in first, 5% in second plus third washings). The Rh polypeptide was subsequently incubated in 1 M hydroxylamine at pH 7 or 9 for 1 h at 22 °C which released >95% of the remaining ^{3}H. The released lipid was partitioned into $CHCl_3$, spotted onto Silica Gel 60 HPTLC plates (Merck), and chromatographed with toluene/methanol/acetic acid (90:10:1) basically as described (28).

RESULTS

Intact human erythrocytes were incubated with [^{3}H]palmitic acid, and the membranes were analyzed by SDS-PAGE and fluorography (Fig. 1). Approximately six major bands were labeled with [^{3}H]palmitic acid, but none corresponded to glycophorins or bands 1–8 (29). A variable amount of the

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[1] The abbreviations used are: SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.

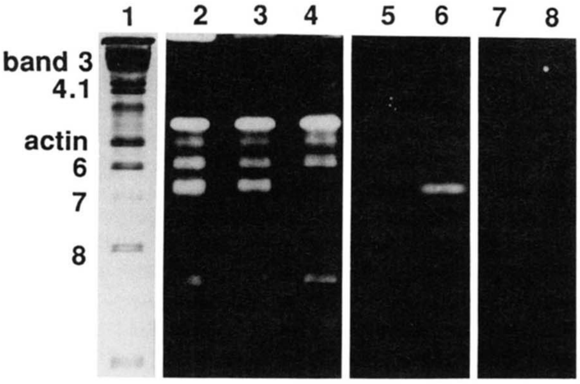

FIG. 1. **[^{3}H]Palmitic acid labeling of the Rh polypeptide and other erythrocyte membrane proteins.** Erythrocytes from the following individuals were labeled with [^{3}H]palmitic acid: an Rh(D)-negative individual, probable *cde/cde* genotype (*lanes 1*, *2*, *5*, and *7*); an Rh(D)-positive individual, *CDe/cDE* (*lanes 3*, *6*, and *8*); and an Rh_{mod} individual (*lane 4*). SDS-PAGE gels of membranes were stained with Coomassie Blue (*lane 1*) or visualized by fluorography (*lanes 2–4*). Polypeptides precipitated from [^{3}H]palmitic acid-labeled erythrocytes with Rh(D) immune globulin (*lanes 5* and *6*) or nonimmune globulin (*lanes 7* and *8*) were analyzed by fluorography. Proteins are identified at the *left margin* (29).

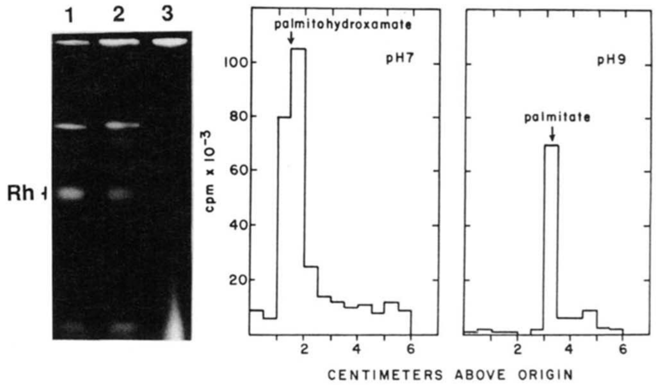

FIG. 2. **[^{3}H]Palmitic acid labeling is most likely through thioester linkages.** The *left panel* is an SDS-PAGE fluorograph of untreated membranes (*lane 1*), $CHCl_3/CH_3OH/H_2O$-extracted membranes before (*lane 2*) and after (*lane 3*) hydroxylamine incubation. Exposure of *lanes 2* and *3* was longer due to lower protein loadings. No qualitative differences of the membrane proteins were noted on Coomassie-stained SDS-PAGE gels. The *center* and *right panels* are thin layer chromatography analyses of fatty acid released from Rh polypeptide by hydroxylamine at pH 7 and 9. *Arrows* identify the locations of standard palmitohydroxamate, R_F = 0.26 (prepared as described, Ref. 36), and free palmitic acid, R_F = 0.42, run on the same plate.

^{3}H label was associated with unidentified material of M_r > 300,000 which remained at the origin of all separating gels. A protein of approximately M_r = 32,000 appeared above band 7 and was consistently heavily labeled, constituting 20–30% of the total ^{3}H label associated with membrane proteins between M_r= 10,000 and M_r = 300,000 when assessed by densitometry.

The M_r = 32,000 band is the Rh polypeptide. A core Rh polypeptide exists in membranes of both Rh(D)-negative and -positive erythrocytes, although the Rh polypeptide in the former lacks the surface D epitope (12). The Rh polypeptide is presumably missing from Rh_{null} erythrocytes and severely diminished in Rh_{mod} erythrocytes, rare phenotypes lacking Rh D, C/c, and E/e antigens. The M_r = 32,000 band was labeled equivalently with [^{3}H]palmitic acid on membranes from Rh(D)-negative and -positive erythrocytes, but the band was labeled extremely weakly on membranes from Rh_{mod} erythrocytes (Fig. 1, *lanes 2–4*). The M_r = 32,000 band was precipitated with Rh(D) immune globulin only from the Rh(D)-positive erythrocytes, but was not precipitated with nonimmune globulin (*lanes 5–8*). Similar bands (not shown) were immunoprecipitated from Rh(D)-positive erythrocytes (CDe/cDE) with monoclonal antibodies specific for the Rh D, c, and E antigens (gift of Dr. J.-P. Cartron, Centre National de Transfusion Sanguine Institut, INSERM U76, Paris).

The [^{3}H]palmitic acid labeling is apparently through thioester linkages. The ^{3}H label associated with membrane proteins was found to survive extraction with chloroform/methanol, but was *trans*-esterified with hydroxylamine (Fig. 2, *left panel*). Rh polypeptide immunoprecipitated from [^{3}H]palmitic acid-labeled erythrocytes was repeatedly washed with chloroform/methanol to elute all noncovalently associated fatty acid. The remaining ^{3}H label (approximately 55% of original) was quantitatively eluted with hydroxylamine at pH 7 or pH 9, and the eluates were analyzed by thin layer chromatography and compared to standards (Fig. 2, *center* and *right panels*). The pH 7 and pH 9 eluates co-chromatographed with standard palmitohydroxamate and free palmitic acid, respectively, indicating that the ^{3}H label represented esterified fatty acid rather than incorporation of a metabolic product derived from the [^{3}H]palmitic acid (30, 31).

Protein synthesis is not required for [^{3}H]palmitic acid labeling. Reticulocytes still actively synthesize proteins, while mature erythrocytes have little or no protein synthesis. [^{3}H]Palmitic acid labelings were compared between peripheral blood from an individual with <1% circulating reticulocytes and another with 23% circulating reticulocytes (Fig. 3, *lanes 1* and *2*). Some differences in labeling other bands were noted, but the overall appearance of the Rh polypeptide was quantitatively and qualitatively very similar. Erythrocytes sedimented in dextran to eliminate all reticulocytes also had a similar labeling pattern (not shown). Incorporation of ^{14}C-amino-acids demonstrated that a small degree of membrane protein synthesis persists in circulating erythrocytes, but not in polypeptides of M_r = 32,000 (Fig. 3, *lane 3*). Protein

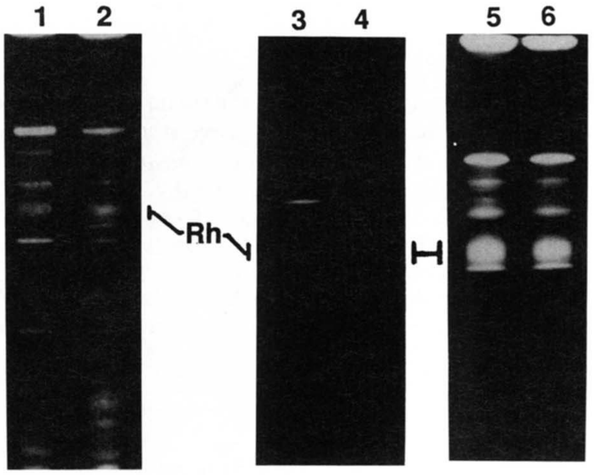

FIG. 3. **Protein synthesis is not required for [^{3}H]palmitic acid labeling of the Rh polypeptide.** Blood was obtained from a normal individual with 0.9% circulating reticulocytes and a patient with sickle cell disease and 23% reticulocytes. Erythrocytes were labeled with [^{3}H]palmitic acid, and the SDS-PAGE gel of the membranes was analyzed by fluorography: normal, *lane 1*; and high reticulocytes, *lane 2*. The effect of protein synthesis inhibition was evaluated by incubating normal erythrocytes with 25 μCi of a mixture of ^{14}C-L-amino-acids (*lanes 3* and *4*) (10 mCi/mmol, Du Pont-New England Nuclear) or [^{3}H]palmitic acid (*lanes 5* and *6*) without (*lanes 3* and *5*) or with 50 μM emetine hydrochloride (*lanes 4* and *6*) (Sigma). The SDS-PAGE gel of the membranes was visualized by fluorography.

synthesis was further reduced to <10% by the addition of emetine (*lane 4*); however, [^{3}H]palmitic acid labeling was not affected (*lanes 5* and *6*).

The association of [^{3}H]palmitic acid with erythrocyte membrane proteins is a continuous process which is fully reversible. [^{3}H]Palmitic acid labeling of the Rh polypeptide and other membrane proteins was detectable after 90 min of incubation and proceeded continuously thereafter when followed for 24 h (Fig. 4, *left panel*). When the labeled erythrocytes were washed and subsequently incubated in an excess of unlabeled palmitic acid, the esterification process was completely reversed (*light panel*). A significant decline in the membrane protein-associated [^{3}H]palmitic acid was observed after only 1 h of chase, and virtually all [^{3}H]palmitic acid was removed after 9 h. It is likely that this represents exchange of the esterified [^{3}H]palmitic acid with unlabeled fatty acid, suggesting that an acylation-deacylation process is continuously ongoing for the Rh polypeptide and a few other membrane proteins. The apparent turnover of the ^{3}H label does not represent degradation of the Rh(D) antigen or other membrane proteins, since Rh(D) immunoreactivity is not reduced after incubation and the Coomassie-stained SDS-PAGE gel from which the fluorograph was prepared demonstrated no detectable degradation of any membrane proteins (not shown).

DISCUSSION

The Rh polypeptide constitutes only 0.5% of the total erythrocyte membrane protein (10, 12), but it is prominently acylated, accounting for 20–30% of the total [^{3}H]palmitic acid linked to membrane proteins in these studies. Fatty acid acylation has been recognized for certain peripheral membrane proteins (32) and on the cytoplasmic domains of certain integral membrane proteins in nucleated cells (33), but the Rh polypeptide is an integral protein largely restricted to the lipid bilayer of erythrocytes (10). It cannot be determined from these studies if the site of the fatty acid acylation of the Rh polypeptide is cytoplasmic, between the leaflets of the bilayer, or on extracellular domains. Compositional studies may permit determination of the number of fatty acids covalently attached to each core Rh polypeptide. The exuberance of the labeling is consistent with multiple acylations, and it has been estimated that each Rh polypeptide contains at least 3–4 cysteine residues (9, 10).

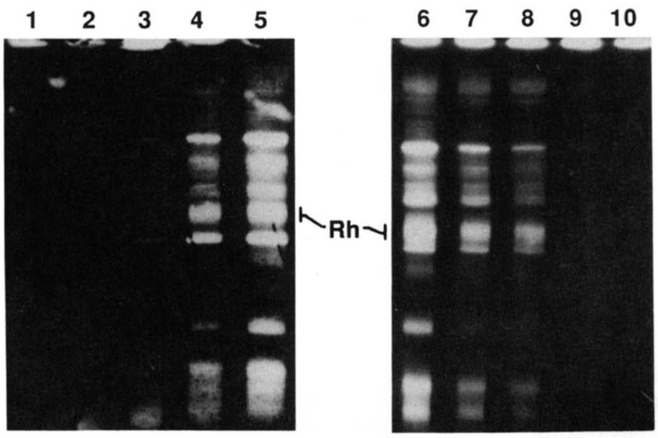

FIG. 4. **[^{3}H]Palmitic acid labeling of erythrocyte membrane proteins is continuous and reversible.** Erythrocytes were labeled with [^{3}H]palmitic acid for various intervals (*lane 1* = 0.5 h, *lane 2* = 1.5 h, *lane 3* = 4.5 h, *lane 4* = 12 h, and *lane 5* = 24 h). The SDS-PAGE gel of the membranes was visualized by fluorography. In a separate experiment, erythrocytes were labeled with [^{3}H]palmitic acid for 5 h, washed free of unadsorbed fatty acid, resuspended in fresh minimal essential medium containing 50 μg/ml unlabeled palmitic acid and incubated for various intervals (*lane 5* = 0 h, *lane 7* = 1 h, *lane 8* = 3 h, *lane 9* = 9 h, and *lane 10* = 19 h). Exposure of the fluorograph of *lanes 6–10* was twice as long as that of *lanes 1–5*.

Covalent attachment of lipid to certain eukaryotic proteins has emerged as an area of intense investigation, but the overall biological importance of these modifications is not well understood (see review, Ref. 23). Likewise, the biological importance of abundant fatty acid acylation of the Rh polypeptide is not obvious, although certain clues may have been uncovered. The Rh polypeptide probably requires specific flanking phospholipid in order to attain the proper conformation on the membrane surface needed for immunogenicity. Early studies demonstrated the requirement of phospholipid for the immunoreactivity of the Rh(D) antigen, since extraction of phospholipid with alcohol resulted in the disappearance of the Rh immunoreactivity which was restored by addition of exogenous phospholipid (15). Rh immunoreactivity is also destroyed by certain phospholipases (16, 17) and can be modulated by enrichment or depletion of membrane cholesterol (18). The overall amino acid composition of the Rh polypeptide includes approximately 50% nonpolar and aromatic residues (9, 10), which is similar to the anion and glucose transporters and other bilayer spanning polypeptides. Nevertheless, the fatty acid acylation appears to be very specific, since these other integral proteins failed to label with the methods employed here.

It is interesting that a single mutation leading to total loss of expression of all the Rh antigens (Rh_{null}) can produce abnormal organization of the phospholipid bilayer (22) and abnormal function of membrane transporters which are embedded within the bilayer (20, 21). Recent work has identified an ATP-dependent membrane enzyme for the maintenance of phospholipid asymmetry (34), and an M_r − 31,000 membrane component has been implicated as a participant (35). Whether the Rh polypeptide plays a role in the active distribution of phospholipid between the leaflets of the bilayer or a passive role in maintaining the bilayer is uncertain. The presence of covalently attached fatty acid side chains may provide the hydrophobic points needed for structurally important protein-phospholipid associations to occur and may be the mechanism through which the Rh polypeptide influences phospholipid bilayer organization.

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